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Electroanalytical determination of diazepam in tablet and human serum samples using multiwalled carbon nanotubes embedded molecularly imprinted polymer–modified carbon paste electrode

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In this study molecularly imprinted polymers were grafted on the surface of functionalized multiwalled carbon nanotubes (MWCNTs) using the diazepam as template molecules. The MWCNTs–MIP were characterized by Fourier transform–infrared spectroscopy (FT–IR) and scanning electron microscopy (SEM) and then used for modification of carbon paste electrode (CPE). Cyclic voltammetry (CV) and square wave voltammetry (SWV) methods were applied to study the binding event and electrochemical behavior of diazepam at the modified carbon paste electrodes. The diazepam binding experiments indicated that the sensor modified by MWCNTs–MIP have much higher adsorption ability than the MWCNTs based non–imprinted polymer (MWCNTs–NIP). Under optimized extraction and analysis conditions, the sensor modified by MWCNTs–MIP exhibited excellent sensitivity(4.0×10^7 μA L $mol^{-1)}$ for diazepam with a linear range of 8.0×10^{-9} to 1.0×10^{-6} mol L^{-1} ($R^2 = 0.9972$) and detection limit of 3.7×10^{-9} mol.L^{-1}. The sensor was successfully applied for determination of diazepam in tablets and human serum samples with recovery values in the range of 91.7–100.2%.

1. Introduction

The determination of drugs in biological samples, mainly in patients' plasma, has been extensively applied to pharmacokinetic studies, in forensic toxicology, to evaluate intoxications by illicit drugs, to assure the therapeutic effectiveness (therapeutically drug monitoring), and to minimize the adverse effects (toxicity symptoms) of prescribed drugs[1].

Diazepam, 7–chloro–1, 3–dihydro–1–methyl–5–phenyl–2H– 1, 4–benzodiazepin–2–one, is the most commonly benzodiazepine drug used as hypnotic, tranquilizer, anticonvulsant and muscle relaxant. It is also an abused drug in which sudden withdrawal, particularly from high dosage, carries the risk of epileptic seizures[2].

A variety of analytical methods have been developed for the determination of this drug in both pharmaceutical and biological samples, such as spectrophotometry[3-6], different chromatographic methods (HPLC, LC/MS, GC, GC/MS, and TLC)[7-11], dispersive liquid–liquid microextraction [12], capillary electrophoresis[2], radioimmunoassay[13] and electrochemical methods[14-19].

Among these methods, electrochemical techniques are a powerful and versatile analytical technique that offers high sensitivity, accuracy, and precision as well as a large linear dynamic range, with relatively low–cost instrumentation than other methods[20].

Selection the type of working electrode materials is an important factor in electrochemical experiments. Carbon-based electrodes are now widely used in electroanalytical chemistry, because of their desirable properties such as availability in a variety of structures, high thermal and mechanical stability, good electrical conductivity, broad potential window, low cost, rich surface chemistry, low background current and chemical inertness. Among the carbon-based electrodes, chemically modified carbon paste electrodes (CMCPEs) have received increasing attention due to their potential applications in different analyses and also its relative ease of electrode preparation and regeneration[21].

Certainly, in addition to sensitivity, selectivity represents the most desired characteristics in an analytical procedure. Challenging problems associated with enhancement of the selectivity and improving the detection limit, are strong incentive to find new materials for electrodes modification[22].

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Molecularly Imprinted polymers (MIP) are promising materials with good selectivity continually being used in electrochemical sensors such as the recognition elements or modifier agents.

MIP is a synthetic polymer possessing selective molecular recognition properties because of its recognition site within the polymer matrix that is complementary to the analyte molecule, regarding the size, shape and positioning of functional groups.

In addition to high selectivity, MIP possess many promising characteristics, such as low cost and easy synthesis, high stability to harsh chemical and physical conditions, and excellent reusability compared to other recognition systems[23].

Nevertheless traditional imprinting techniques most often produce the polymer materials exhibiting high selectivity but low binding capacity, poor site accessibility, and slow binding kinetics[24].

Nanostructured, imprinted materials have a small dimension with extremely high surface to volume ratio, so that most of the imprinted sites are situated at the surface or in proximity of surface. Therefore, the forms of imprinted materials are expected to greatly improve the binding capacity and kinetics and site accessibility of imprinted materials[25].

In the present instance MWCNTs, with high strength, extremely large surface area and unique chemical properties can be considered as the reinforcing element or core in fabricating core–shell structural MIP. Thus, binding sites in the outer layer of the MWCNTs–MIP composite would improve accessibility of the template molecule and reduce binding time [26, 27].

In recent years, various electrochemical sensors based on MWCNTs –MIP composites were reported. For example:

Chena *et al* [28] reports the preparation of MWCNTs functionalized with molecularly imprinted polymers (MIPs) for removal of estrone. An electrochemical sensor fabricated by modifying MWCNTs-MIPs on a glassy carbon electrode surface to recognize dopamine has been reported by Kan *et al* [29]. B.B. Prasad *et al*, were reported a composite of MWCNTs and MIP onto the pencil graphite electrode for trace level detection of insulin [30]. Also a screen printed carbon electrode modified by MWCNTs-MIP composite was used by B.B. Prasad *et al*, for the quantitative analysis of C-reactive protein [31]. A molecularly imprinted poly-methacrylic acid (PMAA), polymerizing on the surface of MWCNTs, was synthesized and used for amperometric detection of uric acid [32].

The MWCNTs–MIP composite can be used as a modifier in preparation of chemically modified carbon paste electrodes. This sensor exhibits both predetermined selective molecular recognition properties and high electrical conductivity. As a result the direct electron transfer to electroactive molecule can be accelerated. In fact, the use of nanotubes as the core in the preparation of MIP can help to the better transfer of electrical current than when MIP are used alone , for modification of carbon paste electrode.

Moreover, electrode surface is easily renewable after smooth polishing it on a paper[33].

To the best of our knowledge, although some researchers studied MIP using diazepam as template[34-41], there is no report regarding preparation of molecularly imprinted polymers on the surface of carbon nanotubes (MWCNTs–MIP) and its use in modification of carbon paste electrode for this molecule.

Therefore, in present study, a composite of MWCNTs and MIP was developed which provide a combination between surface molecular imprinting and nanotechnology. Then we combined advantageous features of DZ selective MWCNTs–MIP nanostructures with carbon paste properties to prepare a cheap, simple, fairly rapid and highly selective and sensitive electrochemical sensor for determination of DZ in complex matrices.

2. Experimental

2.1. Apparatus

Electrochemical data were obtained with a three–electrode system using a 746 VA trace analyzer with 747 VA stand, Metrohm. The modified carbon paste electrode (d=2.5 mm) were used as the working electrodes. A platinum rod and Ag/AgCl electrodes were used as the counter and reference electrodes, respectively. pH measurements were carried out with a 691 pH meter, Metrohm with a combined glass electrode.

Surface morphological images of MWCNTs–MIP composite were recorded using Field Emission Scanning Electron Microscope (FE–SEM), Hitachi, model S–4160. As well as Fourier transform infrared (FT-IR) analysis was carried out on Shimadzu FT-IR–8400s spectrometer (Japan).

2.2. Materials and solutions

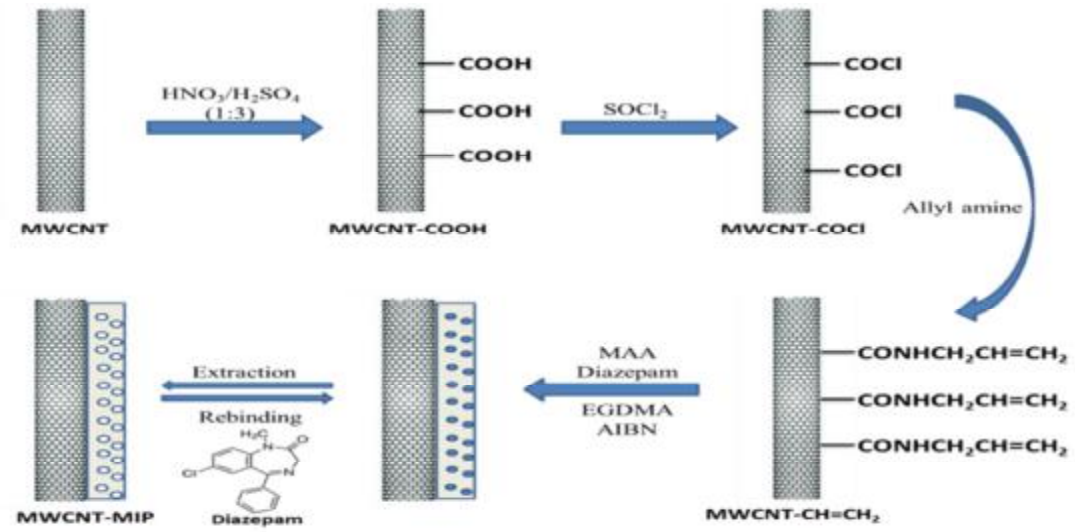
MWCNTs (internal diameter 2–6nm, outer diameter 10–20 nm, length < 30 µm and purity >95%) obtained from Neutrino Corporation (Iran). Methacrylic acid (MAA), Ethylene glycol dimethacrylate (EDMA), Graphite fine powder (spectroscopic grade, particle size < 50 µm) obtained from Merck (Darmstadt, Germany). 2, 2–azobisisobutyronitrile (AIBN) and allylamine were supplied by Sigma–Aldrich (Munich, Germany). Other chemicals were of analytical grade and purchased from Merck. Britton–Robinson (BR) buffer solution with different pH values were used to adjust the pH of sample solutions and supporting electrolyte. The standard stock solution (0.5 mmol L^{-1}) of diazepam was prepared by dissolving the appropriate amount of DZ in ethanol and used to prepare the other concentrations by dilution with BR buffer solution to the mark. All these solutions were maintained under refrigerated conditions in absence of light.

Diazepam tablets containing 10 mg of DZ per tablet was obtained from Sobhan darou (Tehran, Iran) and human blood serum samples were collected from a local pathology laboratory and stored in a refrigerator at ~4 ◦C, before use.

2.3. Modification of MWCNTs

The modification of MWCNTs (Scheme1) is according to the procedure described in reference 26.

For Preparation of MWNTs–COOH, 0.7 g MWCNTs in 200 mL a mixture of H_2SO_4 and HNO_3 (3:1 v/v) solution was ultrasonicated for 4 h, and then the suspension was stirred continuously for 8h at room temperature. The mixture was diluted with deionized water (500 mL) and filtrated through a 0.2µm–pore–diameter nylon membrane. The filtered solid was washed with deionized water



Scheme.1 Protocols for the preparation of MWCNTs–MIP nanocomposite

until the pH was neutral. The resultant black solid (MWNTs–COOH) was then dried under vacuum at 60°C for 24 h.

In order to acylation, 0.6 g of MWCNTs–COOH were suspended in the mixture of 15mL $SOCl_2$ and 45mL chloroform and placed under reflux condition at 60 ◦C for 24 h. Then the excess $SOCl_2$ was removed by multiple washings of the solid with THF and then dried under vacuum to obtain MWCNTs–COCl.

Then to vinylation of MWCNTs, 0.4 g MWCNTs–COCl were dispersed in 40mL THF and then 25mL allylamine, dissolved in 15mL of DMF, was added drop wise to the above mixture. The mixture was stirred at 60 ◦C for 24 h and the solid was filtered and washed with THF. Finally the resulting solid was vacuum dried to obtain vinyl group functionalized MWCNTs (MWCNTs –$CH=CH_2$)

2.4. Preparation of MWCNTs–MIP

In a typical procedure (Scheme1), MWCNTs–MIP were synthesized as follows:

The diazepam (0.4 mmol) and MAA (2.0 mmol) were dissolved in 20 mL of chloroform in a 50.0 mL screw capped glass tube, and was stirred for 1h to preparation of preassembly solution. Subsequently 20 mmol of EGDMA was added into the above solution, stirred for 30 min and followed by addition of 200mg MWCNTs–$CH=CH_2$ (dispersed in 20 mL of chloroform). This mixture was subjected to ultrasound for 30 min to preparation of the prepolymerization solution and then 40mg of AIBN was also added into it. Oxygen was eliminated by purging the mixture with nitrogen for 10 min. Then the glass tube was sealed and cured at 60 ◦C for 24 h. The resultant polymeric particles were washed with chloroform to remove unreacted monomers. Thereafter, the template molecule was removed from the polymer by washing with methanol: acetic acid (8:2, v/v) solution for several times So that no UV–Vis signal is observed for diazepam (at 318 nm) in the eluent. The obtained MWCNTs–MIP were further rinsed with methanol to remove the remaining acetic acid and then dried under vacuum at 60 °C for 24 h before use.

For comparison, multiwalled carbon nanotubes non–imprinted polymers (MWCNTs–NIP) were prepared using the same procedure only without addition of diazepam molecule in the polymerization process.

2.5. Preparation of sensors

For preparation of the bare CPE, n-eicosane as a binder was melted at 45–50 °C and mixed thoroughly with graphite powder in a ratio of 3:1 (w/w). This paste was tightly packed into the end of a glass holder (2.5 mm, *i.d.*) in which electrical contact was made with a copper wire that runs through the center of the electrode body. The excess of solidified material on the electrode surface was removed by polishing it onto a weighting paper until the surface is shiny appearance and then it was rinsed with distilled water. The electrode can be reused after each experiment by cutting a thin layer of paste and polishing of new surface. The modified carbon paste electrodes were prepared in a similar manner, except for adding an appropriate amount of the modifier (MWCNTs–MIP) to the graphite powder.

2.6. General procedure for electrochemical measurements

The modified electrode was incubated into the solution of diazepam in B.R buffer solution (pH=4) for 12 min under stirring with 450 rpm. After that, the electrode was washed (inserted into the washing solution for 6s) and then placed in the electrochemical cell containing 10 mL deaerated (with nitrogen) 0.4 M HCl solution (as the supporting electrolyte). Determination of diazepam was performed by square wave voltammetry method (SWV) using the

pulse amplitude of 50 mV ,scan rate of 20 mV s^{-1} with the frequency of 60Hz over the potential range of −0.45 to −0.85 V versus Ag/AgCl. The results are reported based on triplicate analysis and the average of reduction peak height was used for construction of calibration curve.

2.7. Diazepam assay in real samples

Standard addition method was adapted to determination of DZ in Pharmaceutical formulations (Tablet) and biological (human serum) samples based on triplicate analysis for each concentration.

2.7.1 DZ tablets

For this purpose, ten tablets containing 10 mg of diazepam (Per tablet) were weighed accurately and crushed into a fine powder. A sufficient amount of powder for preparing a stock solution of 1.0×10^{-4} M DZ, was weighed and transferred into 25 mL volumetric flask contained 20 mL ethanol. The content of flask was sonicated for about10 min and then diluted to volume with the same solvent. The solution was next filtrated and desired concentrations of the drug were obtained by accurate dilutions with the BR buffer at pH= 4.0. Finally, these samples were analyzed according to the proposed method described in Section 2.6.

2.7.2 Human serum samples

In order to evaluate the performance of the proposed sensor in biological samples, diazepam –free human serum samples (0.6 mL) were spiked with 0.4 mL of diazepam standard solutions in several concentrations. Then, 0.5 mL acetonitrile was added to the above mixture. After precipitation of plasma proteins, which were isolated by means of centrifuging at 14000 rpm, 0.4 mL of the clear supernatant was transferred into the 25 mL volumetric flask and diluted with the B.R buffer solution (pH=4). Determination of diazepam was carried out by recommended procedure.

3. Results and discussion

3.1. Characterization

3.1.1 Spectral characterization

To confirm the presence of desired functional groups on the surface of MWCNTs and formation of composite between functionalized MWCNTs with MIP, the samples were analyzed by Fourier transform infrared spectroscopy (FT–IR).

Several significant bands in Fig. 1A–b are attributed to –COOH groups introduced on the MWCNTs by acid oxidizing, including the appearance of C=O stretching at 1765 cm^{-1}, C–O asymmetric stretching band at 1126 cm^{-1} and O–H stretching band at 3492 cm^{-1}.

The absorbance at wavenumber of 853 cm^{-1} was assigned to C–Cl stretch vibration, while the absorbance at 1790 cm^{-1} was assigned to C=O stretch vibration for the spectrum of MWCNTs–COCl (Fig. 1A–c).

The following functional groups were identified in the MWCNTs–$CONHCH_2CH=CH_2$ FT–IR spectrum: N–H stretching vibrations (3498 cm^{-1}), C=O stretching vibrations (1793 cm^{-1}), C=C stretching vibrations (1651 cm^{-1}) and –C–N– vibrations (Fig. 1A–d). After the polymerization process for the production of MWCNTs–MIP composite, presence of broad bands at 3446 cm^{-1} corresponding to the N–H and O–H stretching vibrations, a C–H stretching vibrations at 2983 cm^{-1}, a C=O stretching at 1728 cm^{-1}, C–O stretching bands at 1153 cm^{-1} and 1265 cm^{-1} in FT–IR spectra (Fig. A-e) are evidences, which shows that the MWCNTs was successfully grafted with polymers.

3.1.2 Surface characterization

Scanning electron microscopy (SEM) was used to characterize the morphologies of the MIP micro particles, MWCNTs–MIP and MWCNTs–NIP nanocomposites.

As shown in Fig. 1B(middle) the average diameter of the

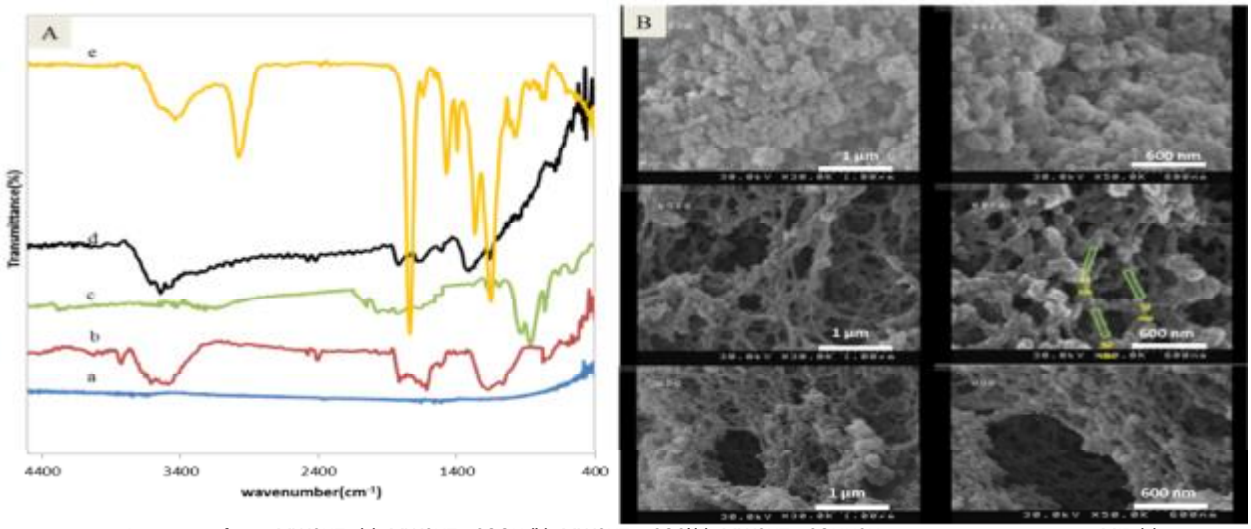


Fig. 1 (A) FT–IR spectra of pure MWCNTs (a), MWCNTs–COOH (b), MWCNTs –COCl(c), MWCNTs–$CONHCH_2CH=CH_2$ (d) and MWCNTs–MIP (e), (B) SEM images of MIP(top), MWCNTs–MIP nanocomposite (middle) and MWCNTs–NIP nanocomposite (down)

MWCNTs–MIP composite is about 50 nm. Since the diameter of purchased nanotubes, was 10 – 20 nm, so the average thickness of the MIP layer on the MWCNTs was about 30 – 40 nm.

Also, Fig. 1B(top) shows that the obtained MIP in absence of MWCNTs have micro-sized dimension. Therefore, formation of the composite between carbon nanotubes and MIP, leading to an increase in surface area and also recognition sites in imprinted polymer.

3.2. Electrochemical behaviour of Diazepam on the bare and modified carbon paste electrodes

Cyclic voltammetry (CV) often is the first experiment to investigate electrochemical behaviour of each compound on the surface of studied electrode. In previous research the electrochemical determination of diazepam often was done by reduction signal of DZ at different electrodes.

Therefore in a preliminary experiment we investigated cyclic voltammetry of 5.0×10^{-5} mol L^{-1} DZ at bare carbon paste electrode during a cathodic scan in the potential range from -0.6 to -1.2 V versus Ag/AgCl electrode in BR buffer solution at pH 4.6.

Fig. 2A shows the cyclic voltammetric response of DZ with the corresponding background voltammogram at scan rate of 100 mV s^{-1}. As can be seen, a single reduction peak at –0.9 V was observed in negative going scan similar to most previous reports[19, 42]. This peak resulted from $2e^-$, $2H^+$ reduction of the 4,5–azomethine group to give 4,5–dihydro–diazepam [17, 43]. No distinct oxidation peak was observed on the reverse scan, indicating to irreversible nature of the reduction process.

In order to achieve improved limits of detection and higher sensitivities, SWV as an appropriate electrochemical technique was used for further investigation. Thus, the response of 5.0×10^{-6} mol L^{-1} DZ on the CP electrode was investigated by SWV method in a 0.05 mol L^{-1} BR buffer (pH= 4.6) (Fig. 2B). This voltammogram (Fig.2B) is also shows a reduction signal of DZ in the potential of -0.86 V.

In order to evaluate the ability of MWCNTs–MIP composite in DZ recognition, the MWCNTs–MIP–CP, MWCNTs–NIP–CP and CP electrodes were prepared and then incubated in 5.0×10^{-6} mol L^{-1} DZ solutions at pH 3.0 for 10 min under stirring.

At this stage, diazepam molecules were selectively bound to the recognition sites in MWCNTs–MIP modified carbon paste electrode. Next, the electrodes were inserted into the BR buffer solution, pH= 3.0 (for 5s) to eliminate any weakly adsorbed and non specifically absorbed analyte and then transferred into a 0.04 mol L^{-1} BR solution with pH 4.6 and the SWV was carried out. Fig.2C illustrates the SWV response of DZ at an unmodified CP, MWCNTs–MIP and MWCNTs–NIP modified CP electrodes. As can be seen, under identical conditions, a well–defined reduction peak (at -0.86V vs. Ag/AgCl) was obtained at the MWCNTs–MIP–CP electrode compared to bare carbon paste electrode. Nevertheless, a small peak for reduction of DZ, was observed at the MWCNTs–NIP–CP electrode indicating non–selective rebinding of DZ with improper sites in the NIP.

Therefore, DZ could be removed from the surface of the MWCNTs–NIP based sensor during the washing process before the determination, whilst in the MWCNTs–MIP based sensor, most of the adsorbed DZ molecules were incorporated in the imprinted cavities through the hydrogen bonding and did not removed easily during the washing process. The entrapment of diazepam in MIP cavities, prevent from quick desorption of this molecule from the electrode surface in the washing and analysis steps.Thus, the MWCNTs–MIP composite as a selective recognition element was used in fabrication of the proposed sensor. Consequently, factors affecting the response of sensor such as polymer features and variables involved in the DZ extraction/ determination processes, were evaluated.

3.3. Effect of the molar ratio of template molecule to functional monomer

Suitable ratio of template molecule, functional monomers and cross–linkers plays a key role on the MWCNTs–MIP characteristics. To achieve a good recognition characteristic, a series of MWCNTs–MIP composite were synthesized with different amounts of diazepam (and so different ratio of DZ: MAA: EGDMA) following the Section 2–4.

The obtained polymers were used for the modification of CP electrodes and response of the sensors (after extraction and washing steps) was evaluated by SWV method. The results (table 1) showed that the optimum molar ratio of template molecule, functional monomers and cross-linkers was 1:5:25.

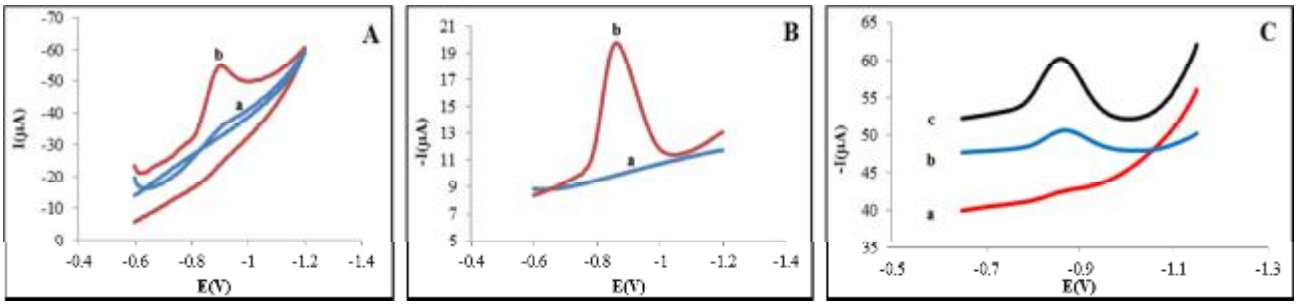


Fig.2 (A) Cyclic voltammetry (scan rate: 100 mV s^{-1}) and (B) SW voltammetry (ΔE= 50 mV, V_{step}= 2 mV, t_{step} = 0.3 s, *Frequency* = 50 HZ) behavior of 5.0×10^{-5} mol L^{-1} diazepam in BR (0.04 M, pH= 4.6) at the surface of bare carbon paste electrode(b) with the corresponding background current(a), (C) SW voltammograms obtained at the (a)CP, (b)MWCNTs–NIP and (c)MWCNTs–MIP modified CP electrodes after incubation the electrodes in 5.0×10^{-6} mol L^{-1} DZ solution for 10 min and recording the voltammograms in BR buffer (pH=4.6).

Table 1 Effect of variation in amount of template molecules used for the preparation of MWCNTs–MIP nanocomposite, on SWV current response

Polymers	Template Diazepam (mmol)	Monomer MAA (mmol)	Cross–linker EGDMA (mmol)	MWCNTs–CH=CH_2 (mg)	Initiator (mg)	–I(μA) (Peak current)
MWCNTs–MIP_1	0.3	2	10	200	40	6.33
MWCNTs–MIP_2	0.4	2	10	200	40	8.02
MWCNTs–MIP_3	0.5	2	10	200	40	6.47
MWCNTs–MIP_4	0.6	2	10	200	40	4.81
MWCNTs–NIP	–	2	10	200	40	2.72
MIP	0.4	2	10	–	40	4.20
NIP	–	2	10	–	40	1.63

Any amount higher than this ratio leads to a decrease in SWV currents apparently due to highly agglomeration of the MWCNTs–MIP composite offering a Poor accessibility of template molecules to the recognition sites. Therefore, 1:5:25 was selected in this work.

As well as to evaluate the effect of nanotubes on the improving the properties of imprinted polymers, MIP and NIP were synthesized in same way but this time in absence of nanotubes. As can be seen in table 1, the use of nanotubes in MIP preparation, leading to an increase in the number of available imprinted sites on the MWCNTs–MIP composite and so significant improvement in response of the MWCNTs–MIP based sensor.

3.4. The effect of washing time on the electrode response

In order to find sufficient washing time to remove the weakly and nonspecifically absorbed DZ molecules from the electrode surface and reproducible response of the sensor, the effect of washing time of the electrode after the extraction step was investigated.
As can be seen in Figure 3, after 4 s, the reduction peak current of DZ in MWCNTs–MIP–CP reaches to a steady state, and it seems that longer washing time does not noticeably decrease the MWCNTs–MIP–CP signal. However, at the same time, the response of the MWCNTs–NIP–CP electrode (Fig. 2(b)) decreases to a large extent by washing. Therefore 6 s was selected as the time taken for the washing step.

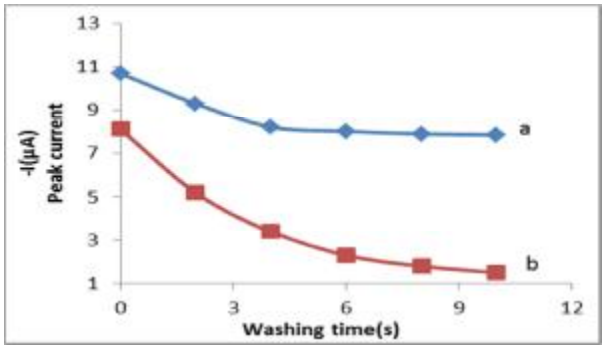


Fig.3 The effect of washing time on the responses of the (a) MWCNTs–MIP–CP and (b) MWCNTs–NIP–CP electrodes.
Extraction solution: 5.0×10^{-6} mol L^{-1} DZ Extraction time: 10 min
SW voltammetry conditions(ΔE= 50 mV, V_{step}= 2 mV, t_{step} = 0.3 s, *Frequency* = 50 HZ) in BR buffer(0.04 M, pH= 4.6)

3.5. Optimization of the MWCNTs–MIP–CP electrode composition

In order to find the best composition for MWCNTs–MIP–CP electrodes, the effects of electrode components which includes MWCNTs–MIP, carbon and n-eicosane, were evaluated. The resulted electrodes at each case were used for diazepam extraction and determination by SWV method. Be experimentally was found that the appropriate amount of n-eicosane to achieve the sensor with suitable physical and electrochemical properties, is 25% (w/w) of total carbon paste composition. Lower than this amount, lead to a decrease in mechanical stability of the electrode and higher values also reduces amount of current response due to insulating effect of the binder.

Thereafter, the MWCNTs–MIP based sensors were prepared in different weight ratios of the MWCNTs–MIP to graphite powder at fixed amount of n-eicosane binder (25 wt %). The results are illustrated in Fig 4l. As can be seen, with increasing amount of MWCNTs–MIP, the reduction peak current after extraction from the 5.0×10^{-6} mol L^{-1} DZ is gradually increased, and it reaches to a maximum at weight ratio of 0.23, due to the increasing in the number of recognition sites on the electrode surface. However, at higher weight ratios than 0.23, the response of sensor is decreased, that may be attributed to decrease in graphite carbon content and consequently decrease in conductivity and electron transfer capability of the paste. Thus, the weight ratio of 61:14:25 for graphite: MIP: binder was chosen as the best composition for the developed MIP sensor performance.

3.6. The effect of different extraction and electrochemical parameters on the sensor performance

By considering this fact that hydrogen interactions are mainly responsible for bonding of template molecules with recognition sites in non–covalent molecular imprinting, the pH of extraction

solution plays an important role on improving response of MIP based sensors. In order to investigate the effect of DZ solution pH, on the DZ extraction, the MWCNTs–MIP–CP electrodes with optimized composition, were immersed into a 5.0 × 10^{-6} mol L^{-1} DZ in BR buffer over the pH range of 2.0–7.0 for 10 min with stirring rate of 300 rpm. After mentioned time, the electrode was washed and immersed into the solution of the electrochemical cell with pH 4.6, followed by square wave voltammetry. The results are shown in Fig. 4II. As can be seen, in pH range of 3.5–4.5, the reduction signal of DZ and hence, the DZ extraction is higher and has no considerable variation in this pH range. At pH values, less than 3.5 and greater than 4.5, the extraction amount tends to decrease.

By consideration of pK_a value of DZ (pK_a =3.3), at low pH range, diazepam exists in cationic form which is not a favourable species for interaction with recognition sites in the MIP, and the extraction of DZ decreases. Similarly, the carboxylic groups situated on the polymer are ionized at pH values higher than pK_a of MAA (pK_a = 4.7) and do not interact with DZ. Therefore, the pH of 4.0 was chosen as an optimum for DZ extraction onto the electrode.

In addition to the extraction pH, effect of supporting electrolyte pH on the electrochemical determination of DZ at pH values ranging from 0.2 to 1.3 (prepared with HCl solution) and 1.6 to 6.0 (prepared with 0.04 M of BR buffer) was investigated. Fig. 4IIIa shows that the catodic peak current is dependent on the pH and decreased with increasing pH. This can be attributed to the fact that the reduction reaction, is H^+ dependent and the acidic media is necessary for this reaction. Thus, in order to obtain high electrochemical responses of the sensor, the acidic pH of about 0.4 was fixed by using HCl (0.4M) solution.

Also, Fig. 4IIIb shows that the potential of reduction peak is pH dependent, throughout the range investigated. However, two different slopes can be seen (63 mV pH^{-1} and 32 mV pH^{-1}) with a break point at pH 3.8. The first slope value of 63 mV pH^{-1}, indicating the participation of an equal number of electrons and protons($2e^-/2H^+$) in the electro–reduction process, which corresponded with previous studies[17, 18]. Moreover, the pK_a of this

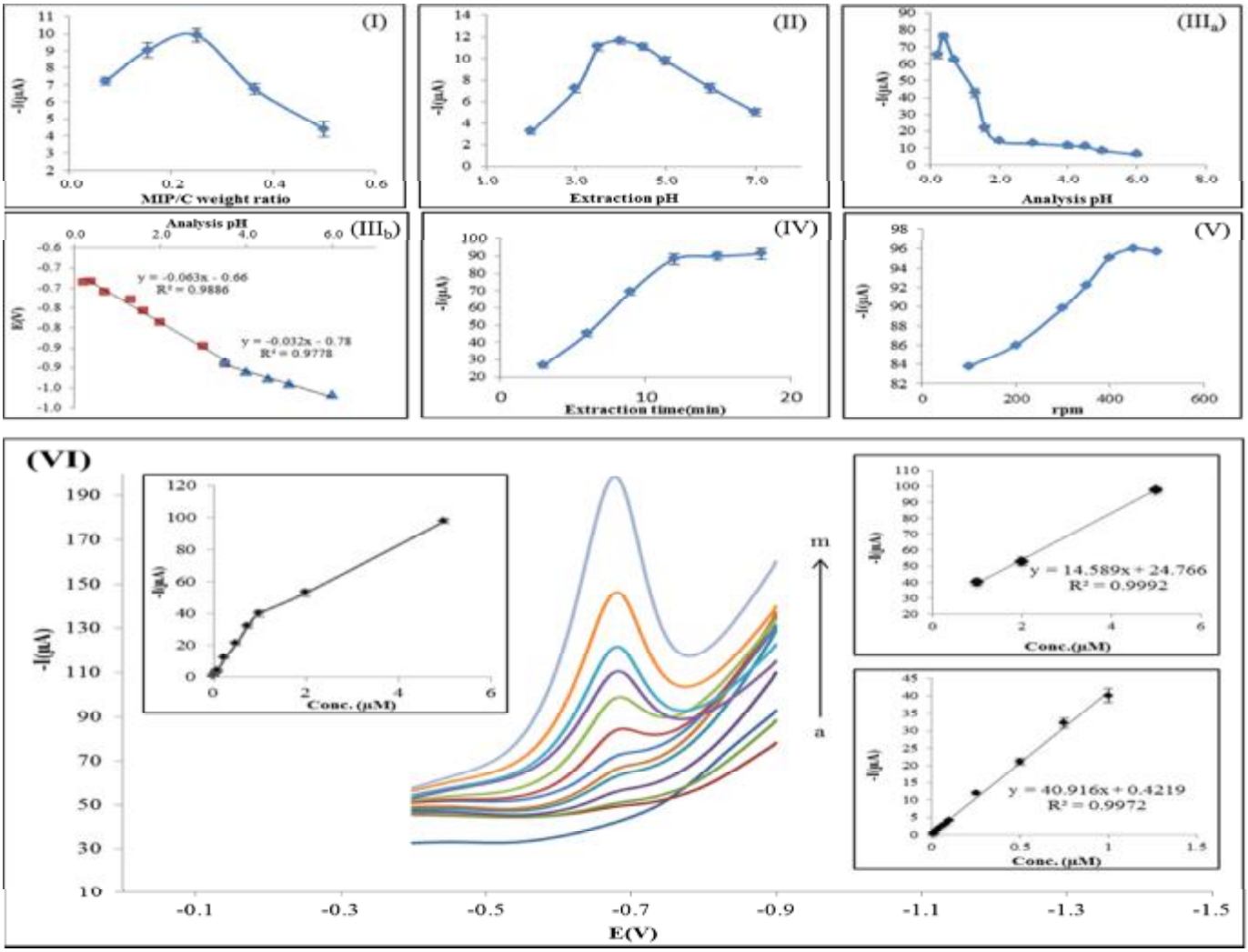


Fig.4 Effect of the (I) MWCNTs–MIP to graphite weight ratio , (II) Extraction pH, (III) pH of supporting electrolyte, (IV) Extraction time and (V) Stirring speed of the extraction solution, on the SWV response of 1.0 × 10^{-6} mol L^{-1} DZ obtained at the MWCNTs–MIP modified CPE. SWV settings: ΔE = 50 mV, V_{step} = 2 mV, t_{step} = 0.3 s, *Frequency* = 50 HZ. (VI) SW voltammograms obtained at the MWCNTs-MIP modified CPE under optimized conditions in the concentration range of 8.0 ×10^{-9} –5.0 ×10^{-6}mol L^{-1} DZ(a to m); inset in (VI): the calibration curve obtained for the developed method and two linear ranges related to it. (-*I*(μA) is peak current of DZ)

drug was found to be near 3.55 associated with protonation of nitrogen atom (N4) of the azomethine group which near the intersection point is obtained in this study.

The effects of extraction time and stirring rate on the diazepam extraction were evaluated (Fig. 4IV and 4V respectively) and found that with an increase of extraction time and stirring rate up to 12 min and 450 rpm, respectively, the SWV response increased and longer extraction times and faster stirring rates, did not considerably affect the diazepam extraction. Therefore, these values were chosen for subsequent experiments.

In order to achieve the highest performance of MWCNTs–MIP based sensor, some of the most important electrochemical parameters related to square-wave voltammetric technique include SW potential amplitude (ΔEs) and SW frequency (f) in the ranges of 10–50 mV and 10–90 Hz were optimized, respectively. By considering the current density and width of SWV reduction peak for sensitive and good profile of voltammetric response, an excellent response was obtained for the pulse amplitude of 50 mV and frequency of 60 Hz.

3.7. Analytical characteristics

After optimization and establishment of method, the SWV response of MWCNTs–MIP–CPE to DZ at different concentrations was studied. As expected, the peak current increased with increasing concentrations of DZ (Fig. 4VI). The calibration curve shows two linear regions. One of these was from 8.0×10^{-9} to 1.0×10^{-6} mol L^{-1} with an equation of I_p (μA) = 40.916 C_{DZ} (μmol L^{-1}) + 0.422 (R^2 = 0.9972), and the other from 1.0×10^{-6} to 5.0×10^{-6} mol L^{-1} with an equation of I_p (μA) = 14.589 C_{DZ} (μmol L^{-1}) + 24.766 (R^2 = 0.9992). The calculated limits of detection (LOD) at MWCNTs–MIP based sensor was 3.77×10^{-9} mol L^{-1} according to the kS_b/m relation, where k = 3 and S_b is the standard deviation of the blank peak currents (n = 7) and m is the slope of the first linear range of calibration curve.

The precision estimated in terms of the relative standard deviation (RSD %) for five repeated measurements of 1.0×10^{-7} mol L^{-1} DZ was 3.4%.

Investigate the sensor stability in determination of 1.0×10^{-7} DZ, showed that the current response of the sensor remained up to 93.1% (RSD=3.7%, n=3) of its initial value after 12 weeks. Table 2 represents the analytical characteristics of the MWCNTs–MIP modified electrode for determination of diazepam.

In order to evaluate the selectivity of proposed sensor, the interference effects of various ions and molecules, on the determination of DZ, were studied. The tolerance limit was defined as the molar ratio of additive/DZ that caused an error less than 5% for determination of 2×10^{-7} mol L^{-1} DZ. At the same time, the influence of similar compounds (i.e. lorazepam, oxazepam and chlordiazepoxide) was also investigated. The obtained results are given in table 3 and show that in the most cases, the performance of developed sensor was not significantly affected by presence of the various species studied.

Table 2 Characteristics of the developed MWCNTs–MIP modified carbon paste electrode for electrochemical determination of DZ by SWV method

Parameter	Value
Linear ranges (mol L^{-1})	8.0×10^{-9} – 1.0×10^{-6} & 1.0×10^{-6} – 5.0×10^{-6}
Slope (μAmol^{-1} L)	40.916
Intercept (μA)	0.422
Correlation coefficient	0.9972
Limit of detection (LOD, mol L^{-1})	3.77×10^{-9}
Precision (RSD%)	3.4
Stability (week)	12

Table 3 Interference study for the determination of 2×10^{-7} mol L^{-1} of DZ under the optimized conditions.

Interferents	Tolerance limit(mol ratio)
Li^+, Na^+, K^+	1000
Mg^{2+},Ca^{2+}	500
CO_3^{2-}, HCO_3^-	500
Cl^-	1000
SO_4^{2-}	100
Glucose	150
Urea	100
Uric acid	30
Ascorbic acid	50
$H_2PO_4^-$	70
Lorazepam	15
Chlordiazepoxide	12
Oxazepam	10

3.8. Analysis of real samples

In order to investigating the capability of the MWCNTs–MIP–CP electrode for determination of DZ in complex matrices, the proposed sensor was applied to determine DZ in tablet and human serum samples according to the procedures described in section 2.7. The results were shown in table 4. As can be seen, good recoveries and RSD% for all samples were obtained revealing that the proposed method has capability to determination of DZ in serum and pharmaceutical samples.

Also, to indicate the accuracy of the method, the results obtained from the developed method was evaluated statistically (by the Student t–test and F–test) as compared with the standard UV–Vis spectrophotometry method according to the USP assay for determination of DZ in tablet.

According to the table 5, at 95% confidence level, $t_{calculated} < t_{theoretical}$.

These results indicate that there is no significant differences between the data obtained using two methods with the accepted value. Furthermore, use of F–test (at 95% confidence level) to compare the precision of two methods, suggests that good performance of the developed method compared to the standard method.

Table 4 Results of DZ determination in real samples (n=3)

Sample	Added (nmol L^{-1})	Detected (nmol L^{-1})	Recovery (%)	RSD %
Tablet	0	49.20 ± 1.04	98.39	2.12
	20	70.14 ± 1.12	100.20	1.59
	100	149.70 ± 1.24	99.80	0.83
	500	550.82 ± 0.82	100.15	0.15
Serum	–	–	–	–
	30	27.50 ± 0.74	91.66	2.68
	150	140.72 ± 1.52	93.81	1.08
	750	694.14 ± 2.54	92.55	0.36

Table 5 Comparison of the developed method, with a standard method for the determination of diazepam in tablet

Method	Standard	Developed
Labeled values (mg)	10	10
Found values (mg)[a]	10.22	9.84
RSD (%)	1.25	2.11
Er_1 (%)[b]	2.20	–1.58
Er_2 (%)[c]	–	–3.70
t value[d]	3.26	1.34
F value[e]	2.66	2.66

[a] n = 3.
[b] Relative error between standard or proposed method and labeled values.
[c] Relative error between the proposed and standard methods.
[d] $t_{theoretical}$ = 4.30.
[e] $F_{theoretical}$ =19.

3.9. Comparison of the developed sensor with other electrodes

The comparison between performance of the developed sensor and other electrodes used for diazepam determination is presented in Table 6. This reveals that the performance of MWCNTs–MIP based sensor is superior or comparable to the reported electrodes in terms of the linear range, detection limit and material used in construction of the sensor.

Table 6 Comparison of some characteristics of the developed sensor and previously reported sensors for DZ determination.

Electrode	Reaction	Technique	Linear range (µmol L^{-1})	Detection limit (µmol L^{-1})	Reference
DME[a]	reduction	LSP[e]	0.056–8.80 & 8.80–200	0.0094	14
CPE	reduction	DPV	0.088–10.5	0.0737	15
LF/GCE[b]	reduction	SWV	0.005–0.492	0.0020	16
Sonogel–Carbon	reduction	SWAdSV	0.098–0.899	0.0140	17
SPE[c]	oxidation	DPAdSV	24.9 – 1001	6.638	17
MWCNTs/CILE[d]	reduction	SWV	0.070–2.67	0.0144	19
MWCNTs–MIP–CPE	reduction	SWV	0.008–1.00 & 1.00–5.00	0.0037	This work

[a] Dropping mercury electrode
[b] Lead film electrode modified glassy carbon electrode
[c] Screen–printed electrode
[d] Carbon nanotube–ionic liquid modified paste electrode
[e] Linear sweep polarography

4. Conclusions

In this research, a highly selective and sensitive modified carbon paste electrode with MWCNTs–MIP was developed for the SWV determination of diazepam at low concentrations. The large surface area of MWCNTs–MIP nanocomposite, increased the number of imprinted sites located on the surface of MWCNTs and their accessibility for detection of analyte, and thus the sensitivity and detection limits of MWCNTs–MIP based sensor is improved. The construction of this sensor is simple and it provides a rapid and economical electrochemical method for the determination of diazepam without any matrix interference in real samples.

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